

**1 New insights into the Manila clam – *Perkinsus olseni* interaction based on gene expression**  
**2 analysis of clam hemocytes and parasite trophozoites through *in vitro* challenges**

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## 34 Abstract

35 The Manila clam (*Ruditapes philippinarum*) is the bivalve species with the highest world  
36 production from both fisheries and aquaculture, but its production is seriously threatened by  
37 perkinsosis, a disease caused by the protozoan parasite *Perkinsus olseni*. To understand the  
38 molecular mechanisms underlying *R. philippinarum*–*P. olseni* interaction, we analyzed the gene  
39 expression profiles of *in vitro* challenged clam hemocytes and *P. olseni* trophozoites, using two  
40 oligo-microarray platforms, one previously validated for *R. philippinarum* hemocytes and a new  
41 one developed and validated in this study for *P. olseni*. Manila clam hemocytes were *in vitro*  
42 challenged with trophozoites, zoospores, and extracellular products from *P. olseni in vitro* cultures,  
43 while *P. olseni* trophozoites were *in vitro* challenged with Manila clam plasma along the same time-  
44 series (1 h, 8 h, and 24 h). The hemocytes showed a fast activation of the innate immune response,  
45 particularly associated with hemocyte recruitment, in the three types of challenges. Nevertheless,  
46 different immune-related pathways were activated in response to the different parasite stages,  
47 suggesting specific recognition mechanisms. Furthermore, the analyses provided useful  
48 complementary data to previous *in vivo* challenges, and confirmed the potential of some proposed  
49 biomarkers. The combined analysis of gene expression in host and parasite identified several  
50 processes in both the clam and *P. olseni*, such as redox and glucose metabolism, protease activity,  
51 apoptosis and iron metabolism, whose modulation suggests cross-talk between parasite and host.  
52 This information might be critical to determine the outcome of the infection, thus highlighting  
53 potential therapeutic targets. Altogether, the results of this study aid to understand the response and  
54 interaction between *R. philippinarum*–*P. olseni* and will contribute for developing effective control  
55 strategies for this threatening parasitosis.

56

57 **Keywords:** Manila clam, perkinsosis, *in vitro* challenge, gene expression, hemocytes, host-parasite  
58 interaction

59

## 60 1. Introduction

61 The Manila clam *Ruditapes philippinarum* (Veneridae; Adams and Reeve, 1850) is the  
62 bivalve mollusk with the highest world production and a major contributor to the livelihoods of  
63 many coastal communities in Europe and Asia. However, the sustainability of Manila clam  
64 production is threatened worldwide by perkinsosis, a disease caused by the parasite *Perkinsus*  
65 *olseni*. Over the years, perkinsosis mass mortality events have been recorded in Manila clam both in  
66 Asia (Liang et al., 2001; Wu et al., 2011; Nam et al., 2018; Waki et al., 2018) and Europe (Sanmartí  
67 et al., 1995; Pretto et al., 2014). Despite perkinsosis seriously concerns clam farmers, no treatment  
68 or effective preventive measures are currently available for this disease.

69 The life cycle of *P. olseni* includes four developmental stages, namely zoospore,  
70 trophozoite, prezoosporangium (hypnospore) and zoosporangium, all involved in the direct  
71 transmission of perkinsosis from host to host (Villalba et al., 2004). Zoospore is a biflagellated free  
72 swimming stage that transforms into trophozoite once it has invaded the host (Wang et al., 2018).  
73 Trophozoite is the most observed stage in the host, where it vegetatively proliferates; trophozoites  
74 released from the host, either through diapedesis or in the feces, are able to infect other clams  
75 (Villalba et al. 2004); furthermore, trophozoites give rise to prezoosporangia in moribund hosts  
76 (Villalba et al., 2004; Casas and La Peyre, 2013). In seawater, prezoosporangia develop into  
77 zoosporangia, where hundreds of zoospores are produced and eventually released (Casas et al.,  
78 2002a).

79 Several studies have been carried out to understand the response of Manila clam to *P. olseni*  
80 and the lesions associated with the disease (reviewed in Soudant et al., 2013); particularly profuse  
81 are those highlighting the role of the hemocytes, multifunctional cells of the hemolymph which  
82 infiltrate the infected tissue. Recently, the genetic response of Manila clam hemocytes to  
83 perkinsosis has been characterized through high-throughput transcriptomic sequencing  
84 (Hasanuzzaman et al., 2017) and microarray analyses either after *in vivo* challenge with  
85 trophozoites (Romero et al., 2015) or zoospores, as well as wild exposure (Hasanuzzaman et al.,

86 2018). Furthermore, the protein profiles of clam hemocytes and plasma after *in vivo* challenge with  
87 zoospores have also been reported (Fernández-Boo et al., 2016).

88 Clam perkinsosis has also been studied from the parasite point perspective focusing on  
89 different biological processes, including its proliferation (Casas et al., 2002b; Elandalloussi et al.,  
90 2003, 2005a; Araujo et al., 2013), metabolic pathways (Elandalloussi et al., 2005b), pathogenicity  
91 (Shimokawa et al., 2010; Waki et al., 2012; Waki and Yoshinaga, 2013), population genetic  
92 structure (Pardo et al., 2011; Vilas et al., 2011), and gene and protein expression profiles (Ascenso  
93 et al., 2007; Leite et al., 2008; Ascenso, 2011; Fernández-Boo et al., 2014, 2015a, 2015b).  
94 Moreover, the transcriptome of *P. olsenii* trophozoites challenged with Manila clam plasma has been  
95 recently assembled and annotated (Hasanuzzaman et al., 2016). As described for the congener  
96 species *Perkinsus marinus* (Villalba et al., 2004; Pales Espinosa et al., 2014), the extracellular  
97 products released by *P. olsenii* appear to be involved in the pathogenesis of the disease (Fernández-  
98 Boo et al., 2014, 2015b). However, to our knowledge, the differential response of hemocytes to the  
99 different *P. olsenii* life stages and its extra-cellular products has not been investigated, and no  
100 studies have provided an integrated view of the host-parasite interaction. Host resistance and  
101 parasite virulence are intricately intertwined, and thus their responses should be studied in parallel  
102 to thoroughly understand the host-parasite interaction (Nelson, 1973; Day, 1974).

103 High throughput technologies, including microarrays and RNA-Seq, are commonly used to  
104 study the genetic responses underlying host-parasite interactions (Coyne et al., 2011; Hughes et al.,  
105 2011; Manque et al., 2011; Leite et al., 2013). Microarrays have become a universal tool for  
106 analyzing the expression of thousands of genes (Bubendorf, 2001; Allison et al., 2006; Sobek et al.,  
107 2006) and have been used in the past to understand different biological processes in Manila clam  
108 (Milan et al., 2011; Moreira et al., 2012, 2014; Allam et al., 2014; Menike et al., 2014; Romero et  
109 al., 2015). Functional information might be combined with other studies addressed to identify  
110 molecular markers useful for breeding programs. For example, selection to increase resistance of  
111 *Crassostrea virginica* against diseases, including the infection with the congener species *P.*

112 *marinus*, have been successfully implemented in the USA (Frank-Lawale et al., 2014; Proestou et  
113 al., 2016; Casas et al., 2017).

114 Here, we developed and validated the first microarray platform for *P. olsen*i trophozoites,  
115 which was applied, along with the previously developed for *R. philippinarum* hemocytes  
116 (Hasanuzzaman et al., 2018), to analyze host-parasite interaction *in vitro* across a time series (1 h, 8  
117 h, and 24 h) under different scenarios: a) *P. olsen*i trophozoites challenged with *R. philippinarum*  
118 plasma; and b) clam hemocytes challenged with *P. olsen*i trophozoites, zoospores and extracellular  
119 products. The results obtained complement the information from previous studies of perkinsosis in  
120 Manila clam and provide new insights on the processes underlying *R. philippinarum*-*P. olsen*i  
121 interaction. This knowledge is critical to devise successful disease prevention strategies.

122

## 123 **2. Materials and methods**

### 124 **2.1. Experimental design and sampling**

125 *R. philippinarum* collected from a *P. olsen*i-free area (Camariñas, NW Spain) were used to  
126 collect hemocytes for the *in vitro* challenge with *P. olsen*i trophozoites, zoospores and extracellular  
127 products (proteins released into the culture medium by *P. olsen*i trophozoites). Similarly, *P. olsen*i  
128 trophozoites were challenged *in vitro* with *R. philippinarum* plasma collected from clams of the *P.*  
129 *olseni*-free area. The details for collection of hemocytes, trophozoites, zoospores and extracellular  
130 products have been described in Hasanuzzaman et al. (2017). The procedures to collect trophozoites  
131 from *in vitro* cultures (1-2 months old) and the isolation of Manila clam plasma have been described  
132 in Hasanuzzaman et al. (2016). Absence of *P. olsen*i infection in every used clam was confirmed by  
133 PCR and incubation of gill pieces in Ray's fluid thioglycollate medium. All experiments were  
134 carried out in the facilities of Centro de Investigaci3n Marinas (CIMA; Spain).

135

#### 136 **2.1.1. *In vitro* challenge of clam hemocytes with *Perkinsus olsen*i**

137 *R. philippinarum* hemocytes ( $5 \times 10^6$ ) were challenged *in vitro* with *P. olsen*i trophozoites  
138 ( $5 \times 10^6$ ), zoospores ( $5 \times 10^6$ ) and extracellular products (2.5 mL of culture media enriched with  
139 extracellular products) separately in IWAKI 6-well plates (Fig.1A). Each challenge included three  
140 biological replicates for both treatment and control (only culture media) groups, and each biological  
141 replicate was a pool of hemocytes from 10 different clams, thus averaging individual biological  
142 variation. For the challenges, trophozoites and zoospores, obtained just before the challenge, were  
143 separately suspended in 2.5 mL filtered seawater (FSW) and added into a permeable insert (0.2  $\mu$ m  
144 Anopore® membrane NUNC 25 mm) in each well. For hemocyte-extracellular products challenge,  
145 2.5 mL of culture media enriched with hemocyte extracellular products were added into the inserts  
146 of the respective wells. The inserts allowed the flow of media but not the cells; hence, hemocytes  
147 and parasite cells were never in contact. Samples for RNA extraction were collected at 1, 8 and 24 h  
148 after the start of the challenge. Further details are available in Hasanuzzaman et al. (2017).

149

#### 150 **2.1.2. *In vitro* challenge of *P. olsen*i trophozoites with Manila clam plasma**

151 *P. olsen*i trophozoites ( $\sim 5 \times 10^6$ ) resuspended in 2.5 mL FSW were placed in IWAKI 6-well  
152 plates, and 2.5 mL of plasma (treatment) or FSW (control) were added into a permeable insert (0.2  
153  $\mu$ m Anopore® membrane NUNC 25 mm) set in the plate-wells (Fig. 1B). Four pseudo-replicates  
154 (trophozoites from the same culture) for both control and treatment groups were collected at 1, 8  
155 and 24 h since the onset of the challenge. Further details are available in Hasanuzzaman et al.  
156 (2016).

157

#### 158 **2.2. RNA extraction**

159 Total RNA was extracted using Qiagen RNeasy mini kit with DNase treatment following  
160 manufacturer's instructions. The RNA quality and quantity were evaluated in a Bioanalyzer (Bonsai  
161 Technologies) and a NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies Inc)  
162 respectively.

163

## 164 **2.3. Microarray analyses**

### 165 **2.3.1. *R. philippinarum* hemocyte microarray experiment**

166 The design of the *R. philippinarum* oligo-microarray has been described and validated in  
167 Hasanuzzaman et al. (2018). Briefly, this microarray was designed on an 8 × 15 k Agilent format  
168 and included 14,621 probes representing 11,052 transcripts of which 10,813 were annotated  
169 (97.8%).

170 Thirty-two microarrays (four slides) were used. *R. philippinarum* control replicates at each  
171 time point were pooled, so a single control microarray was used for each sampling time (1-C, 8-C,  
172 24-C); in addition, the low RNA amount in the controls of the zoospore challenge determined  
173 pooling all controls in a single microarray hybridization.

174

### 175 **2.3.2. *P. olsenii* trophozoite microarray design and experiment**

176 To construct the *P. olsenii* oligo-microarray, we selected 10,104 sequences from our  
177 previously published *P. olsenii* trophozoite transcriptome (Hasanuzzaman et al., 2016). A total of  
178 9,369 sequences were selected because they were annotated to transcripts in the NCBI nr protein  
179 database, while the remaining 735 non-annotated sequences were selected by their notable  
180 differential expression in our preliminary evaluation (Hasanuzzaman et al., 2016). One oligo-probe  
181 was designed for annotated sequences (known sense) and two probes (sense and antisense) were  
182 designed for the non-annotated sequences. We also included 4,158 technical replicates for  
183 microarray reproducibility evaluation. All processes for oligo-probe design and Agilent oligo-  
184 microarray procurement were similar to those followed for the Manila clam microarray  
185 (Hasanuzzaman et al., 2018).

186 A total of 16 microarrays (two slides) were used for the experiment. A single pooled control  
187 was hybridized in two microarrays per slide as technical replicates. Twelve microarrays were used  
188 for the treatments across the time-series (1 h, 8 h and 24 h) including four replicates per time point.



190 **2.3.3. Hybridizations and analysis**

191 Hybridizations were performed at the Universidade de Santiago de Compostela (USC) Functional  
192 Genomics Platform using the Agilent Technology Gene Expression Unit following a one-color gene  
193 expression analysis protocol. All hybridizations were carried out by the same researcher in the same  
194 day. Hybridized slides were scanned using an Agilent scanner (G2565B, Agilent Technologies) and  
195 signals were captured and processed. The microarray platforms (Agilent-072098 and Agilent-xxx)  
196 and data presented in this publication has been deposited in the NCBI's Gene Expression Omnibus  
197 (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and are available under accession number xxxx.  
198 Microarray hybridization, data processing and quality filtering were carried out as previously  
199 described (Hasanuzzaman et al., 2018). Briefly, the gProcessedSignal value was used for statistical  
200 analyses, and the spatial detrend correction was applied using the Agilent Feature Extraction  
201 algorithm. Normalization within each microarray was carried out applying the loess method. After  
202 data quality evaluation, only probes with absolute fluorescence values > 200 were considered robust  
203 for further microarray analyses. We determined the variation and reproducibility associated with  
204 both biological and technical replicates by estimating Pearson correlation coefficients. Further,  
205 Spearman's Rho correlation between identical probes for the same gene was estimated across all  
206 microarrays.

207 Hierarchical Clustering, Principal Component Analysis (PCA), Self Organizing Tree  
208 clustering Algorithm (SOTA) and other statistical analyses, such as *t*-test and ANOVA were  
209 performed using MultiExperiment Viewer version 4.9.0 (tMev) of the TM4 Microarray Software  
210 Suite (Saeed et al., 2006). Differentially expressed genes (DEGs) between treatment and control  
211 samples were detected with a *t*-test using a false discovery rate (FDR) of 0.05 (SAM analysis).  
212 DEGs between different challenges and times were detected using a two-way (for *R. philippinarum*  
213 microarray) and one-way (*P. olseni*) ANOVA. DEGs were functionally characterized with Gene  
214 Ontology (GO) terms using Blast2GO Version 3.2 (Conesa et al., 2005) with default parameters.

215 Enriched GO terms were determined by comparing the set of DEGs in a comparison with the full  
216 set of annotated transcripts of either Manila clam or *P. olsenii* using Blast2GO Fisher's exact test  
217 (FDR = 0.05).

218

## 219 **2.4. Microarray validation**

220 Quantitative PCR (qPCR) was performed to validate the results of both *R. philippinarum*  
221 hemocyte and *P. olsenii* trophozoite microarrays. Some samples had not enough RNA for qPCR  
222 after microarray hybridization, and therefore 22 out of 25 and 8 out of 12 microarrays were  
223 validated for *R. philippinarum* and *P. olsenii*, respectively. Moreover, since the remaining *P. olsenii*  
224 control RNA quantity was not enough for qPCR analysis, we used as control one of the treatment  
225 replicates of 1h that showed a highly significant positive correlation ( $r = 0.85$ ) with control  
226 microarrays and further, it was positioned very close to controls in the PCA analysis and far away  
227 from the two other 1 h treatment biological replicates. A significant and high positive correlation  
228 was detected between qPCR and microarray data in the *P. olsenii* trophozoites challenge (see  
229 Results) supporting our strategy.

230 To select genes for validation, we followed the random stratified procedure proposed by  
231 Miron et al. (2006) with some modifications as described by Millán et al. (2011). Log2 fold change  
232 (FC) variation of each probe across all experimental conditions was determined and the standard  
233 deviation (SD) estimated. Taking into account the SD range and gene annotation (preferably  
234 immune-related genes), we selected 14 and 7 genes for qPCR validation in the Manila clam and *P.*  
235 *olsenii* experiments, respectively. Thereafter, FCs of these genes were ordered in ascending values  
236 for the 22 *R. philippinarum* microarrays (14 genes  $\times$  22 microarrays = 308 cases) and the 8 *P. olsenii*  
237 microarrays (7 genes  $\times$  8 microarrays = 56 cases), and then subdivided into a series of strata. A total  
238 of 39 cases were selected in the *R. philippinarum* experiment considering strata FC range, and at  
239 least one gene per microarray and one case per gene. For *P. olsenii*, 19 cases were selected  
240 considering FC range, with at least three genes per microarray and one case per gene. Two

reference genes were selected considering their least expression variation across microarrays and literature suggestions (Filby and Tyler, 2007; Infante et al., 2008): 60S ribosomal protein L18 (*RPL18*) and 40S ribosomal protein S12 (*RPS12*) for Manila clam, and 60S ribosomal protein L12 and 40S ribosomal protein S3 for *P. olsenii* qPCR validation.

Primers were designed using *Primer Express* Software v2.0 (Applied Biosystems) with default settings (Table S1). Primer specificity to the PCR template (i.e. selected gene) was checked using NCBI/Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>). The qPCR analysis was conducted as described by Hasanuzzaman et al. (2018).

### 3. Results

#### 3.1. Quality of microarray data

The *R. philippinarum* hemocyte microarray fluorescence values exhibited a very high reproducibility between technical replicates within microarrays ( $r > 0.99$ ,  $p < 0.001$ ). Correlation between biological replicates was also high ( $r > 0.88$ ,  $p < 0.01$ ). Hierarchical clustering and PCA analyses showed appropriate clustering of biological replicates at each time point in all experiments. After removing those probes with absolute fluorescence values  $< 200$ , the number of remaining probes per microarray ranged from 4,295 to 5,961.

Similarly, the *P. olsenii* microarray showed high reproducibility ( $r > 0.98$ ,  $p < 0.01$ ; Pearson correlations for absolute fluorescence values between replicates). The hierarchical clustering and PCA analyses showed sample clustering according to the time point series, except for one of the replicates at 1 h, which as previously mentioned (See Material and Methods section 2.4) clustered close to controls. After removing probes with absolute fluorescence values  $< 200$ , the number of remaining probes was 6,500, 6,493 and 6,679 for 1, 8 and 24 h respectively.

#### 3.2. Validation of microarray data by quantitative real-time PCR

266 Twelve genes showed a significant correlation between *R. philippinarum* hemocyte  
267 microarray and qPCR data ( $r = 0.68$ ;  $p < 0.01$ ), and there were no significant differences between  
268 both datasets ( $t = 0.860$ ;  $p = 0.397$ ). The ubiquitin B and serine protease genes both demonstrated  
269 late amplification and dissociation curves with multiple peaks when analyzed by qPCR and  
270 therefore were excluded from further analyses.

271 The expression of selected *P. olseni* genes by qPCR and microarray was also significantly  
272 correlated ( $r = 0.76$ ;  $p < 0.01$ ) and no significant differences were found between both datasets ( $t =$   
273  $1.570$ ;  $p = 0.134$ ).

274 The significant correlation of microarrays and qPCR for the selected genes supports the  
275 consistency of our microarray platforms. The minor discrepancies might be explained by the lower  
276 RNA quality of some samples, the putative existence of chimaeras in our transcriptome databases  
277 derived from the bioinformatics assembling pipeline, and the existence of paralogous genes and/or  
278 splice variants; genomic expansion of gene families has been observed in bivalves, such as the  
279 expansion of stress-related genes in Pacific oyster *Crassostrea gigas* (Zhang et al. 2012). Resulting  
280 paralogous genes could complicate the interpretation of microarray results due to cross-  
281 hybridization.

282

### 283 **3.3. Gene expression profiles in *R. philippinarum* hemocytes**

#### 284 **3.3.1. Differential expression between control and *P. olseni*-challenged hemocytes**

285 The number of DEGs varied greatly across the time-series and in response to the different *P.*  
286 *olseni* stages (Fig. 2; Table S2). Further, the response of hemocytes to the different challenges was  
287 rather specific with few common up- or down-DEGs (Fig. 3). The highest number of DEGs were  
288 shared between extracellular products and trophozoite challenges at 24 h, while nearly 50% DEGs  
289 (up- and down-regulated) were shared between extracellular products (8 h) and trophozoites (1h)  
290 datasets (Fig. S1). On the other hand, the response to zoospores was very specific; the highest

291 number of DEGs was detected at 1 h, mostly up-regulated, while nearly only down-regulated genes  
292 were detected at 8 h (207 of 208 DEGs) and only 4 DEGs were found at 24h.

293 The top DEGs ( $FC > |3|$ ) for the three challenges (Tables 1, 2 and 3) were mostly time-  
294 specific. DEGs encoding different structural components of ribosomes, NADH dehydrogenase  
295 subunit 4 (*ND4*) and ubiquitin isoform cra\_e were detected in the three types of challenge. Some  
296 stress- and immune-related genes, such as those from the family of cytochrome p450 (e.g. *CYP2H2*-  
297 like, *CYP18A1*, *CYP2J2*-like), complement c1q-like protein 4 (*CIQL4*), serum amyloid a (*SAA*) and  
298 ras-related c3 botulinum toxin substrate 2 (*RAC2*) were found among the top DEGs in the  
299 hemocytes challenged with trophozoites and zoospores; however, their modulation was less intense  
300 but still significant in the challenge with the extracellular products (Table S2). Other genes, such as  
301 tandem repeat galectin (*TRGal*) or apolipoprotein d (*APOD*), were also significantly modulated in  
302 all the scenarios (Table S2), although only appeared as top DEGs in one of the challenges.

303 The GO-term analyses rendered several enriched functions presumably related to the Manila  
304 clam defense response in the three challenges (Fig. S2). GO terms related to the activation of innate  
305 immune response, particularly hemocyte recruitment (vascular endothelial growth factor receptor  
306 signaling pathway, positive regulation of macrophage chemotaxis), were found in the three  
307 challenges at 1h. Endopeptidase activity, redox processes, iron metabolism, protein synthesis,  
308 cytoskeleton structure and mitosis/cell proliferation were found enriched in more than one  
309 challenge.

310 Other recurrent GO terms showed a particular pattern regarding the challenge and/or the  
311 time; GO-terms related to the toll-like receptors and MAPK signaling pathways were up-regulated  
312 in response to the zoospores at 1 h, but down-regulated in the challenges with the extracellular  
313 products at 8h and the trophozoites at 24 h. A similar pattern was also observed for NIK/NFkB and  
314 RAS transductional signaling, both up-regulated in hemocytes challenged with zoospores at 1 h, but  
315 down-regulated in response to extracellular products at 8 h. Moreover, the GO term negative  
316 regulation of apoptotic processes was down-regulated at 8 and 24 h in response to extracellular

317 products and trophozoites, respectively, while monooxygenase activity was up-regulated in both  
318 cases either at 8 h or 24 h.

319 The analysis also identified some enriched functions that appeared challenge-specific; the  
320 up-regulation of genes involved in cell junction assembly and platelet aggregation in response to  
321 extracellular products, the activation of natural killer cell mediated cytotoxicity processes in the  
322 case of the challenge with trophozoites, or that of the TNF signaling in response to zoospores.

323

### 324 **3.3.2. Differences between hemocytes challenged with different *P. olsenii* stages**

325 A two-way ANOVA analysis detected 2,996 DEGs between challenges (trophozoites,  
326 zoospores or extracellular products; Fig.4, Tables S3, S4, S5) and 2,014 DEGs between times (1 h,  
327 8 h, 24 h; Fig. 5; Tables S6, S7, S8). There was a strong differential response of hemocytes to the  
328 different *P. olsenii* stages or extracellular products (Table S3). A self Organizing Tree Algorithm  
329 (SOTA) analysis identified a total of nine clusters of DEGs showing highly correlated expression  
330 profiles across challenges ( $r > 0.9$ ) (Table S4, Fig. S3A). GO-term enrichment was analyzed within  
331 each SOTA cluster (Table S5). In group P-SG4 (Fig. 4), GO terms related to redox processes and  
332 endopeptidase inhibitory activity were enriched, and genes appeared to be down-regulated in  
333 response to extracellular products at 8 h, while up-regulated in response to trophozoites at 24 h. In  
334 group P-SG8, genes related to cysteine-type peptidase activity were found again up-regulated in the  
335 trophozoite challenge at 24 h, while down-regulated in response to zoospores (Fig. 4). Indeed, the  
336 enriched GO terms of groups P-SG8 and P-SG9 revealed that, starting from 8 h, the hemocytes  
337 challenged with zoospores showed a notable down-regulation of genes related to the immune  
338 response (chemokine activity and chemotaxis processes), defense response (apoptosis signaling  
339 pathway, mitosis), as well as cellular structure (actin filament and actomyosin structure  
340 organization) and metabolism (glycolysis and gluconeogenesis, ATP synthesis, lipid binding) (Fig.  
341 4). Moreover, GO terms related to protein synthesis, extracellular matrix (laminin receptor, focal  
342 adhesion), stress (ubiquitin activity) and phosphatidylinositol-mediated signaling were enriched in

343 group P-SG6, characterized by genes up-regulated in the extracellular products challenge at 24 h,  
344 but slightly down-regulated in response to trophozoites and zoospores (Fig. 4).

345 We also analyzed the expression profile of the 50 more discriminating genes between  
346 challenges to look for particular and combined patterns that could aid to characterize the different  
347 parasite stages across the time-course (Fig. S4). These genes precisely distinguished the hemocyte  
348 response by challenge and time since all replicates were clustered within the same group.  
349 Extracellular products challenge was grouped in a single cluster close to trophozoite challenge,  
350 while zoospore challenge was clustered in a differentiated group related to trophozoite 8 h. The  
351 clustering of these 50 genes showed four main gene profiles separated by horizontal bars in Figure  
352 S4. Regrettably, a certain amount of these genes could not be consistently annotated, which limits  
353 their utility, but several stress response and apoptosis-related genes were identified characterizing  
354 stages / times across infection. A future analysis and annotation of these genes will aid to  
355 understand the response to the different parasite stages involved in the infection process.

356

### 357 **3.3.3. Time-specific gene expression in IVT challenged hemocytes**

358 The 2,014 DEGs showing significant differences across times (two-way ANOVA; Fig.5;  
359 Table S6) were split into nine subgroups of genes with highly correlated patterns ( $r > 0.9$ ) using the  
360 SOTA algorithm (Table S7, Figure S5). Two of these groups (T-SG2, T-SG3) showed quite similar  
361 expression patterns in hemocytes in response to extracellular products and trophozoites, while a  
362 different pattern was observed in response to zoospores (Fig. 5, Table S7). These genes, up-  
363 regulated in response to extracellular products and trophozoites at 8 h, were enriched in specific GO  
364 terms: heme binding, related to iron metabolism, and respiratory chain (group T-SG2, Table S8,  
365 Fig. 5A), or GO-terms related to immune response, apoptosis and cytoskeleton (group T-SG3;  
366 Table S8; Fig. 5B). Gene expression for these two groups showed an opposite pattern in the

367 zoospore experiment at 1 h, being down-regulated in T-SG2 and up-regulated in T-SG3, while, by  
368 contrast, their expression was reduced at both 8 and 24 h.

369

### 370 **3.4. Gene expression in *P. olsenii* trophozoites challenged with Manila clam plasma**

371 The FC values of *P. olsenii* trophozoites challenged with Manila clam plasma ranged  
372 between -2.4 and +3.9 (Table S9). A total of 65 DEGs were detected at 1 h (42 up- and 23 down-  
373 regulated), 48 at 8 h (37 up- and 11 down-regulated), and 300 at 24 h (201 up- and 99 down-  
374 regulated) (Fig. 6). At 24 h most DEGs (46%) were either not annotated or annotated as  
375 hypothetical proteins (Table S9). GO-term annotation revealed that most up-regulated genes at 1 h  
376 were associated with stress-response, iron-sulfur cluster assembly and cell-redox homeostasis, while  
377 most down-regulated genes were related to proteolysis. Oxidation-reduction processes and  
378 proteolysis appeared again among the overrepresented functions at 8h and 24 h. At 8 h, there was a  
379 clear trend showing the genes associated to oxidation-reduction processes among those up-  
380 regulated, while proteolysis-related genes were down-regulated. By contrast, most proteolysis-  
381 related genes were up-regulated at 24 h, as well as several genes associated with glycolysis, directly  
382 involved in cell redox homeostasis (e.g. gamma-enolase, glyceraldehyde-3-phosphate  
383 dehydrogenase (*GAPDH*), fructose-1,6-bisphosphate aldolase (*FBA*)).

384 A one-way ANOVA ( $p < 0.05$  with standard Bonferroni correction) identified a total of 886  
385 DEGs along the time series (Table S10). The SOTA clustering algorithm identified two groups of  
386 genes (Table S11, Fig. S5). The first group (258 genes) included genes up-regulated at 1 h and  
387 down-regulated at 24 h, while the second (628 genes) presented an opposite pattern. This last group  
388 showed a significant enrichment in multiple GO terms related to DNA replication (Table S12),  
389 likely associated with imminent cell proliferation.

390

## 391 **4. Discussion**



392 In this study, we analyzed the interaction between Manila clam and *P. olsenii* through an *in*  
393 *vitro* time course experiment using hemocytes and plasma of the host, and different life stages and  
394 extracellular products of the parasite. This study complements our previous *in vivo* challenge  
395 (Hasanuzzaman et al., 2018) and aids to dissect the genetic interaction between the parasite and the  
396 hemocytes at the initial stages of infection.

397 Several genes, such as *SAA*, *ND4*, *CYP450*, cathepsins and *APOD*, suggested as potential  
398 biomarkers in the *in vivo* challenge (Hasanuzzaman et al., 2018) were also found differentially  
399 expressed in all the scenarios here analyzed, most of them among the top DEGs in one or more  
400 challenges. This confirms their key role during perkinsosis and their potential for further  
401 investigations aimed to achieve perkinsosis-resistant strains in selective breeding programs. These  
402 genes are involved in processes previously identified as relevant in the pathogenesis of perkinsosis,  
403 such as innate immunity (*SAA*), cell redox homeostasis and reactive oxygen species (ROS)  
404 production (*ND4* and *CYP450*), apoptosis and lysosomal degradation processes (cathepsins) and  
405 lipid metabolism (*APOD*) (Leite et al., 2013; Soudant et al., 2013; Romero et al., 2015;  
406 Hasanuzzaman et al., 2018). *SAA* is among the most powerful acute-phase proteins in vertebrates,  
407 being involved in immune-cell chemotaxis and cytokine secretion (Badolato et al., 1995; Ribeiro et  
408 al., 2003), and these functions as mediators of the inflammatory response appear to be conserved in  
409 bivalves (Rosani et al., 2016). Cell migration and infiltration of hemocytes in parasitized tissues  
410 mediated by chemo-attractant stimuli had been previously reported during perkinsosis infected  
411 clams (Villalba et al., 2004; Soudant et al., 2013). In Manila clam, the recruited hemocytes are  
412 responsible for the encapsulation of the parasite (Montes et al., 1995a, 1995b), which would then  
413 be attacked and destroyed by the synergistic effects of ROS and lysosomal enzymes (Soudant et al.,  
414 2013). Another gene involved in ROS production whose expression was strongly modulated in the  
415 different challenges was *RAC2*. This gene encodes a small Rho GTPase necessary for NADPH  
416 oxidase full assembly, but it is also involved in actin cytoskeleton organization occurring in  
417 chemotaxis and phagocytosis (Tell et al., 2012). The down-regulation of *RAC2* in response to

zoospores and trophozoites at different times deserves further work to investigate its role during perkinsosis, considering the multiple evidences indicating that *Perkinsus* spp. is able to interfere with the phagocytic capacity of hemocytes as well as its certain tolerance to ROS (Soudant et al., 2013). Another protein related to Rho GTPases, namely Rho GTPase activating protein 6, had been pinpointed as a possible marker for resistance to *P. olsenii* (Fernández-Boo et al., 2016).

Our study also highlights the role of lectins and C1q domain containing proteins in perkinsosis, both involved in innate immunity through pathogen cell recognition and agglutination processes (Kishore et al., 2004; Kim et al., 2008a, 2008b); particularly, the C1q-like lectin *CIQL4* and *TRGal* were differentially expressed in the three challenges. The activation of lectin complement pathway via C1q-like lectins seems to be a key mechanism in the innate immunity of Manila clam in response to *P. olsenii* infection (Prado-Alvarez et al. 2009; Leite et al., 2013, Hasanuzzaman et al., 2018). Likewise, cytoskeleton organization was found modulated in response to the different challenges, mostly through up-regulation of genes at early stages (1 h) in response to zoospores and through down-regulation at later times, when in turn their expression increased in the challenges with trophozoites and extracellular products. This process plays an essential role in the response of immune-related cells such as hemocytes (May and Machesky, 2001), but host cytoskeleton is also a common and recurring target for the infectious strategies of pathogenic microbes (Gruenheid and Finlay, 2003). Modulated expression of cytoskeleton-related genes was previously reported in perkinsosis-infected Manila clam (Romero et al., 2015; Fernández-Boo et al., 2016; Hasanuzzaman et al., 2017, 2018).

The response of hemocytes to zoospores was the most differentiated one in our study, particularly at 1 h, where the most intense modulation was detected. Comparing the *in vitro* response of hemocytes challenged with zoospores with that previously observed *in vivo* (Hasanuzzaman et al., 2018), we found several common enriched GO terms between 1 and 10 h post challenge. Particularly interesting is the enrichment of MAPK activity, toll-like receptors and NIK/NFκB signaling pathways among up-regulated genes, which in turn appeared among those

444 down-regulated at different time points in the challenges with trophozoites and extracellular  
445 products in the present study.

446 Mitogen-activated protein kinases (MAPK) signal cascades, characterized as the most  
447 ancient and evolutionarily conserved signaling pathways (Widmann et al., 1999), are involved in  
448 cell growth, apoptosis, inflammation and response to environmental stresses. One of the well-  
449 studied downstream components of the MAPK signaling pathway is the nuclear transcription factor  
450 kappa B (NFκB), directly involved in regulating different immune functions (Akanda and Park,  
451 2017; Sun, 2017). As well, toll-like receptors (TLRs) act as primary sensors that detect a wide  
452 variety of microbial components by the recognition of pathogen-associated molecular patterns  
453 (Allam and Raftos, 2015), and TLR signaling pathway plays pivotal roles in host innate immune  
454 defense mechanism. The downstream signaling after pathogen detection by TLRs includes the  
455 activation of MAPK and NFκB, which regulate the expression of cytokines, chemokines and  
456 interferons that ultimately protect the host from microbial infection (Kawasaki and Kawai, 2014).  
457 This kind of response was only found in the hemocytes challenged with zoospores in our study and,  
458 together with the expression of cytoskeleton-related genes, suggests a different mechanism of  
459 recognition between zoospores and trophozoites that would activate different immune-related  
460 pathways in the host. Later on, a broad down-regulation was found in the zoospore challenge at 8 h,  
461 involving genes related to a variety of relevant cell functions, while almost no DEGs were found at  
462 24 h, reflecting a return to a homeostasis state. Further work is needed to elucidate if this pattern  
463 could be induced by *P. olsenii* as a strategy associated with its transformation from zoospore into  
464 trophozoite, which appears to occur soon after host invasion, but the mechanisms involved are not  
465 clear yet (Wang et al., 2018).

466 As expected, we found more similarities between the hemocyte response to trophozoites and  
467 their extracellular products. Particularly, the response against extracellular products at 8 h was  
468 clustered with the response against trophozoites at 1 h (~ 50% shared DEGs), thus it appears to be  
469 delayed, although the analyses indicated that both responses start to converge at 24 h. The most

470 relevant mechanisms that were similarly regulated in these challenges were redox and glucose  
471 metabolism, protease activity, apoptosis and iron metabolism. The integrated analysis of the  
472 changes in the transcriptome of both the hemocytes and the trophozoites of *P. olsenii* allowed us to  
473 gain more insight into these functions involved in Manila clam - *P. olsenii* interaction.

474 In host-parasite interactions, metabolic-related genes are usually significantly regulated; the  
475 parasite requires energy for its survival, growth and reproduction, while the host needs energy to  
476 elicit the immune response and cope with the infection. These bioenergetic needs of cells are met by  
477 the interconnected pathways of glycolysis, tricarboxylic acid cycle and oxidative phosphorylation  
478 (Ganeshan and Chawla, 2014). Particularly, up-regulation of glycolysis is a critical step in the  
479 activation of immune cells, to synthesize macromolecules and generate the antimicrobial respiratory  
480 burst (Ganeshan and Chawla, 2014). ROS production is an important microbicidal mechanism of  
481 hemocytes, but, if it is not well counter balanced by their cellular antioxidant capacity, leads to  
482 oxidative stress, which is often associated with the pathogenesis of infectious diseases (Ray et al.,  
483 2012). We found that pathways related to oxidation-antioxidation were regulated in both the  
484 parasite and the host, particularly active in hemocytes challenged with trophozoites at 24 h,  
485 confirming a critical role in their interaction, as suggested in our previous studies (Hasanuzzaman et  
486 al., 2016, 2017, 2018). Evidence of oxidative stress and activation of antioxidant defenses by  
487 hemocytes included the modulation of *APOD*, which might have a role as an antioxidant molecule  
488 during perkinsosis (Hasanuzzaman et al., 2018), but also *TXNDC*, acting in thioredoxin system, or  
489 glutathione S-transferases, *GSTs*. Up-regulation of antioxidant-related genes was also detected in *P.*  
490 *olsenii* trophozoite, such as *AHPC*, a member of family peroxidases involved in controlling  
491 endogenous and exogenous peroxides in response to stress (Hofmann et al., 2002), or *GST*,  
492 peroxiredoxin 5 and membrane selenoprotein, previously related to antioxidant activity in *Perkinsus*  
493 spp. (Araujo et al., 2013, Fernández-Boo et al., 2014, 2015a, 2015b). On the other hand, the down-  
494 regulation in the trophozoite of prostatic acid phosphatase precursor might hinder the ability of *P.*  
495 *olsenii* to deal with ROS, since this gene has been reported to play a role in the eradication of

496 hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and the inhibition of O<sub>2</sub><sup>-</sup> in *P. marinus* (Volety and Chu, 1997; Soudant  
497 et al., 2013; Pales Espinosa et al., 2014).

498 Interestingly, some DEGs that act in redox and glucose metabolism detected in both the host  
499 and the parasite, such as enolase, *GAPDH* and *FBA* are also known to provide function as  
500 plasminogen receptors (Yang et al., 2010; Ghosh and Jacobs-Lorena, 2011; González-Miguel et al.,  
501 2013), and might be involved in enhancing the virulence of the parasite. Pathogens are capable of  
502 modulating host cells to acquire and / or recruit plasminogen / plasmin, which can degrade  
503 immunoglobulin, complement molecules and extracellular matrix proteins (Jolodar et al., 2003;  
504 Chung et al., 2011), thus facilitating adherence, evasion of the immune response, tissue penetration  
505 and migration, and nutrition uptake (Kitt and Leigh, 1997; Jolodar et al., 2003; Yavlovich and  
506 Rottem, 2007; Siemens et al., 2011; Figuera et al., 2013). Particularly, *GAPDH*, has been shown to  
507 inhibit complement C3 activity, modulating the immune response of the host of the parasite  
508 *Haemonchus contortus* (Sahoo et al., 2013). In this study, the expression of different genes related  
509 to plasminogen metabolism was modulated in both the trophozoites and hemocytes, particularly in  
510 the hemocytes challenged with extracellular products. In that challenge we found down-regulation  
511 of various complement components at 8 h and 24 h, and enriched GO terms related to extracellular  
512 matrix at 24 h (DEGs found by the ANOVA analysis between challenges). This could be also  
513 associated with the enrichment of the platelet aggregation signaling pathway (directly related with  
514 plasminogen / plasmin) found in the hemocytes against extracellular products at 1 h and 8 h.  
515 Platelets play a prominent role in tissue repair and regeneration in vertebrates, with relevant  
516 connection with several pathways of immune response and apoptosis (Gawaz and Vogel, 2013). In  
517 invertebrates, hemocytes serve as multipurpose defense cells, which include a role in the processes  
518 of tissue repair and remodeling (Cerenius and Söderhäll, 2010). The early up-regulation of the  
519 platelet aggregation pathway could then be a response induced by the tissue damaging factors  
520 contained in extracellular products, responsible for the characteristic tissue degradation observed in  
521 perkinsosis (Pales Espinosa et al., 2014; Fernández-Boo et al., 2015b). A potential challenge-

522 specific response can be found in the down-regulation of genes related to phosphatidylinositol  
523 biosynthesis and protein glycosylation, also detected at 24 h, possibly aimed at the inhibition of  
524 trophozoite proliferation by reducing glycosylated-phosphatidylinositol anchors, which  
525 demonstrated to be essential for survival in several parasite species (Martin and Smith, 2006;  
526 Ferguson et al., 2017).

527 On the other hand, the enrichment of the GO term “natural cell mediated cytotoxicity  
528 processes” was the most characteristic result in the hemocytes challenged with trophozoites at 1 h.  
529 This suggests the triggering of a cytotoxic response against that parasite stage by the hemocytes,  
530 that were previously reported to present this natural killer-like activity in different invertebrates  
531 (Franceschi et al., 1991; Parrinello, 1996; Chernysh et al., 2004). The different onset of the  
532 hemocyte response against the trophozoite and extracellular products might underlie the different  
533 timing observed in the regulation of redox and glucose metabolism, as well as apoptosis and  
534 proteases activity.

535 Cathepsins-related genes, whose expression appeared widely regulated in this study, are  
536 responsible for driving proteolytic degradation within the lysosome and in the extralysosomal  
537 milieu, and under certain conditions, such as ROS production, they are released in the cytoplasm  
538 and participate in the execution of apoptosis (Chwieralski et al., 2006; Repnik et al., 2012). This has  
539 been considered a relevant host defense mechanism against the infection by *Perkinsus* spp (Soudant  
540 et al., 2013; Romero et al., 2015), and in this study we found several evidences of apoptosis  
541 modulation. Particularly, the expression of cathepsins and apoptosis-related genes was mostly up-  
542 regulated in the hemocytes challenged with trophozoites at 1 and 24 h and in those challenged with  
543 extracellular products at 8 and 24h. Nevertheless, we found some evidences that *P. olseni* might  
544 interfere with the apoptotic machinery as a strategy to facilitate its proliferation by suppressing  
545 host-cell apoptosis at certain stages of the infection, as reported for other parasitosis (Keller et al.,  
546 2006; Sokolova, 2009; Gervais et al., 2018). In fact, some apoptotic genes were inhibited in  
547 hemocytes after being exposed to *P. olseni* stages, particularly in the challenge with zoospores

548 starting from 8h, after their initial up-regulation at 1 h. Also, the up-regulation of Bax inhibitor in *P.*  
549 *olseni* trophozoite and the down-regulation of *BCL2* in the hemocytes challenged with trophozoites  
550 at the same time point (24 h) might represent a direct interaction of Bax inhibitor with *BCL2* to  
551 modulate apoptosis in host cells (Xu and Reed, 1998).

552 The expression of several genes encoding cathepsins and other proteolysis-related genes was  
553 also modulated in *P. olseni* trophozoites, mostly down-regulated at early stages and up-regulated at  
554 24 h, when the parasite showed the most intense transcriptomic changes. It is well known the  
555 importance of proteases as virulence factors, involved in the invasion, immune evasion, nutrition,  
556 and reproduction of parasites (Armstrong, 2006; Lilburn et al., 2011). This has been also proved for  
557 *Perkinsus* spp. in a study related to *P. marinus* virulence (Pales Espinosa et al., 2014) and by the  
558 presence of proteolytic factors amongst *P. olseni* extracellular products (Fernández-Boo et al.,  
559 2015b). On the other hand, the host response involves the production of protease inhibitors that are  
560 capable of inactivating and clearing the proteases involved in parasitic invasion (Armstrong, 2006).  
561 These inhibitors have demonstrated to play a key role for resistance in some cases, such as the  
562 serine protease inhibitor cvS-1 of oysters against *P. marinus* (La Peyre et al., 2010). This issue still  
563 remains to be elucidated in Manila clam-*P. olseni* interaction; in our work, we did not find a clear  
564 increase in the expression of protease inhibitors in challenged hemocytes, although a potential  
565 candidate could be alpha macroglobulin (up-regulated in the challenge with *P. olseni* trophozoites),  
566 which is an important component of the invertebrate innate immune response capable of binding  
567 and neutralizing the diverse array of proteases that function as virulence factors (Armstrong, 2010).

568 Finally, it is well-known the relevance of iron metabolism during protozoa infections, where  
569 a constant battle between the host and the invader around this element, essential for parasite  
570 survival, has been reported (Weinberg, 2009). Iron availability was demonstrated to be involved in  
571 the proliferation, virulence and metabolic pathways of *P. olseni*, being iron metabolism proposed as  
572 a promising therapeutic target (Elandalloussi et al., 2003, 2005a; Leite et al., 2008; Araujo et al.,  
573 2013). In this work, several genes involved in iron metabolism were regulated in the hemocytes,

again with a similar expression pattern in challenges against trophozoites and extracellular products (up-regulated), while their down-regulation was observed in response to zoospores at 1 h post-challenge. The most relevant DEGs were those encoding ferritins, the main iron-storage proteins, whose increased gene expression indicates an iron withholding defense system (Weinberg, 2009), and proteins involved in the biogenesis and assembly of iron-sulfur (FeS) clusters, such as *ISCA1* (Cózar-Castellano et al., 2004). FeS clusters are among the most ancient and versatile protein cofactors, playing both structural and catalytic roles, acting in central metabolic processes such as electron transfer, redox chemistry, enzyme catalysis, and sensing environmental or intracellular conditions to regulate gene expression (Beinert et al., 1997; Dellibovi-Ragheb et al., 2013). In a previous study on the transcriptome of *P. olsenii* stimulated with clam plasma, Hasanuzzaman et al. (2016) reported the modulation of genes encoding FeS assembly protein, and here we also found in challenged trophozoites at 1 h several up-regulated genes involved in this pathway. These included the iron-sulfur cluster assembly protein *SUFB*, a member of the sulfur mobilization system, a pathway of the FeS biogenesis machinery that is present in plastid-containing organisms, such as the apicomplexan parasites, where it is sequestered to the apicoplast organelle (Dellibovi-Ragheb et al., 2013). There are multiple evidences indicating that *Perkinsus* spp., closely related to Apicomplexa, contains a plastid organelle (Joseph et al., 2010). In particular, *de novo* isoprenoid synthesis, a characteristic process occurring in that organelle, was detected in both *P. marinus* and *P. olsenii* (Matsuzaki et al., 2008; Hasanuzzaman et al., 2016). The SUF pathway in the plastid would be responsible for supplying FeS clusters to proteins involved in isoprenoid synthesis, a process strongly suggested to be essential for the viability of parasites, making it an attractive candidate for the development of new drug targets (Dellibovi-Ragheb et al., 2013).

596

## 597 **5. Conclusions**

598       The comparison of the different Manila clam - *P. olsenii* cell/product challenges across the  
599 time course has provided new useful data to understand the host-parasite interaction during



perkinsosis. Globally, the analyses revealed a fast response of the hemocytes to *P. olsenii*, modulating a substantial number of genes involved in innate immune responses, but *P. olsenii* zoospores, trophozoites and extracellular products showed to trigger differential transcriptomic changes in hemocytes, especially connected with the early activation of different immune-related pathways. Our results aid to understand the hemocyte response to the different parasite stages, including their interaction with parasite virulence factors throughout the infection process. Moreover, the relevance of several genes previously indicated as potential biomarkers for perkinsosis was confirmed here, and the integrated analysis of host and parasite transcriptomic changes highlighted the main mechanisms involved in this host-parasite interaction, providing new candidate molecules and therapeutic targets to control perkinsosis in Manila clam.

610

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621

## 622 **Supplementary data**

Supplementary data associated with this article can be found, in the online version, at <https://data.mendeley.com/datasets/zvnw69chcz/draft?a=b53e943a-aec2-4646-ad4b-3f3646b13c13>

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## 932    **Legends to Figures**

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934    **Figure 1.** Experimental design of *in vitro* challenges. (A) Manila clam hemocytes (H) vs  
935    *Perkinsus olseni* trophozoites (T), zoospores (Z) and extracellular products (ECP) from *in vitro*  
936    cultured parasites; (B) *P. olseni* T vs Manila clam plasma. FSW= filtered seawater. RFTM =  
937    Ray's fluid thioglycollate medium.

938    **Figure 2.** Number of differentially expressed genes in Manila clam hemocytes (H) challenged  
939    with *Perkinsus olseni* extracellular products (ECP), trophozoites (T) and zoospores (Z) along a  
940    time series. Up- and down-regulated genes are shown above and below the horizontal axis,  
941    respectively, and their intensity with a colour degree from light to dark according with their FC.

942    **Figure 3.** Venn diagrams showing the number of down- and up-regulated genes across the time  
943    course in Manila clam hemocytes (H) challenged with *Perkinsus olseni* extracellular products  
944    (ECP), trophozoites (T) and zoospores (Z).

945    **Figure 4.** Most relevant functionally enriched SOTA groups from Manila clam hemocytes (H)  
946    differentially expressed genes (Parasite Factor; ANOVA,  $p < 0.001$ ) after challenging with different  
947    *Perkinsus olseni* parasite stages/products (ECP = extracellular products, T = trophozoites and Z =  
948    zoospores) between challenge types. A) P-SG4; B) P-SG6; C) P-SG8; D) P-SG9.

949    **Figure 5.** Most relevant functionally enriched SOTA groups from Manila clam hemocytes (H)  
950    differentially expressed genes (Time Factor; ANOVA,  $p < 0.001$ ) after challenging with different  
951    *Perkinsus olseni* parasite stages/products (ECP = extracellular products, T = trophozoites and Z =  
952    zoospores) across a time series. A) T-SG2; B) T-SG3.

953    **Figure 6.** Differentially expressed genes in the *in vitro* stimulated *Perkinsus olseni* trophozoites (P-  
954    T) challenged with Manila clam plasma through a time series. Up- and down-regulated genes are  
955    shown above and below the horizontal axis, respectively, and their intensity with a colour degree  
956    from light to dark according with their FC.

957

## 958 **Legends to Supplementary Files**

959

960 **Figure S1.** Venn diagrams comparing differentially expressed genes of *Perkinsus olseni* hemocytes  
961 against trophozoite (H-T) at 1h post challenge vs. extracellular products (ECP) at 8h post challenge;  
962 A) down-regulated genes; B) up-regulated genes; C) all genes.

963 **Figure S2.** Gene Ontology (GO) term enrichment of up- and down-regulated genes in *R.*  
964 *phillipinarum* hemocytes after *in vitro* challenges with *Perkinsus olseni* extracellular products  
965 (ECP), trophozoites (T) and zoospores (Z) at 1 h, 8 h and 24 h post-challenge.

966 **Figure S3.** Nine SOTA groups including highly correlated gene profiles identified among the  
967 differentially expressed genes of *Perkinsus olseni* hemocytes between challenges using a two-way  
968 ANOVA. **B.** Nine SOTA groups including highly correlated gene profiles identified among the  
969 differentially expressed genes across the time series using a two-way ANOVA.

970 **Figure S4.** Hierarchical clustering of differentially expressed genes in Manila clam hemocytes  
971 (Parasite Factor, ANOVA,  $p < 0.001$ ) after challenging with different *Perkinsus olseni* parasite  
972 stages/products (ECP = extracellular products, T = trophozoites and Z = zoospores).

973 **Figure S5.** SOTA groups including highly correlated gene expression profiles among the  
974 differentially expressed genes of *Perkinsus olseni* trophozoites after a challenge with *R.*  
975 *phillipinarum* plasma using a one-way ANOVA.

976 **Table S1.** qPCR primers used to validate *Perkinsus olseni* microarray data.

977 **Table S2.-** Differentially expressed genes of *R. phillipinarum* hemocytes (FDR = 5 %; FC > 1  
978 or < -1) after challenges with several *Perkinsus olseni* forms across a time series (1h, 8h,  
979 24h).



980 **Table S3.-** Differentially expressed genes ( $p < 0.001$ ;  $1 < FC < -1$ ) between hemocyte  
981 *Perkinsus olseni* challenges (extracellular products, ECP; trophozoites, T; and zoospores, Z)  
982 across the time series (1 h, 8 h, 24 h) using a two-way ANOVA (factor challenge).

983 **Table S4.-**Nine groups of hemocyte differentially expressed genes (factor *Perkinsus olseni*  
984 form, ANOVA,  $P < 0,001$ ) showing a significant correlation pattern ( $r > 0.90$ ; SOTA groups).

985 **Table S5.-**Enriched GO terms in the nine SOTA groups identified among the differentially  
986 expressed genes detected in hemocytes between *Perkinsus olseni* challenges (ANOVA,  $p <$   
987  $0.001$ ).

988 **Table S6.-** Differentially expressed hemocyte genes ( $p < 0.001$ ;  $1 < FC < -1$ ) after *Perkinsus*  
989 *olseni* challenges between times (1 h, 8 h, 24 h) using a two-way ANOVA (factor time).

990 **Table S7.-**Differentially expressed genes of hemocytes after *Perkinsus olseni* challenges in  
991 the three most relevant SOTA groups (factor time, ANOVA,  $P < 0,001$ ) showing a significant  
992 correlation pattern (SOTA groups) in the experimental conditions analyzed.

993 **Table S8.-**Enriched GO terms in the three most relevant SOTA groups of differentially  
994 expressed genes of hemocytes (factor time, ANOVA,  $P < 0,001$ ) after *Perkinsus olseni*  
995 challenges showing a significant correlation pattern (SOTA groups) in the experimental  
996 conditions analyzed.

997 **Table S9.-** Differentially expressed genes of *Perkinsus olseni* trophozoites ( $FDR = 5 \%$ ;  $FC >$   
998  $1$  or  $< -1$ ) after challenge with Manila clam plasma along a time series (1h, 8h, 24h).

999 **Table S10.-** Differentially expressed genes of *Perkinsus olseni* trophozoite after a challenge  
1000 with Manila clam plasma across a time series using a one-way ANOVA ( $p < 0,05$  after  
1001 Bonferroni correction)

1002 **Table S11.-**Two groups of trophozoites differentially expressed genes (ANOVA,  $P < 0,05$   
1003 after Bonferroni correction) after challenge with Manila clam plasma showing a significant  
1004 correlation pattern ( $r > 0,90$ ; SOTA groups).

1005 **Table S12.-** Enriched GO terms in the two SOTA groups of differentially expressed genes of  
1006 trophozoites (ANOVA,  $P < 0,05$ ) after challenge with Manila clam plasma showing a  
1007 significant correlation pattern (SOTA groups) in the experimental conditions analyzed.

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1009

**Table 1.** Top differentially expressed genes (FC > |3|) annotated to known proteins in the Manila clam hemocytes challenged with *Perkinsus olseni* extracellular products.

Annotation	GO terms	Log <sub>2</sub> FC*		
		1 h	8 h	24 h
NADH dehydrogenase subunit 4	C:mitochondrion; F:NADH dehydrogenase (ubiquinone) activity; P:ATP synthesis coupled electron transport; P:oxidation-reduction process	-	4.6	-
Alpha amylase	F:hydrolase activity, acting on glycosyl bonds; P:carbohydrate metabolic process	-	4.1	-
60s ribosomal protein l26	C:large ribosomal subunit; F:structural constituent of ribosome; P:translation	-	3.9	-
50s ribosomal protein l14	C:large ribosomal subunit; F:structural constituent of ribosome; P:translation	-	-	3.9
Type i inositol -trisphosphate 5-phosphatase 12 related	F:inositol-polyphosphate 5-phosphatase activity; P:protein dephosphorylation	-	3.9	-4.4
Asparagine synthetase	F:asparagine synthase (glutamine-hydrolyzing) activity; P:glutamine metabolic process; P:negative regulation of apoptotic process	-	3.2	-
Protein odd-skipped-related 2 isoform x2	F:nucleic acid binding; P:positive regulation of cell proliferation	-	-	3.1
40s ribosomal protein s15aa	C:ribosome; F:structural constituent of ribosome; P:mitotic spindle elongation	-	3.0	-
Cartilage matrix	C:collagen trimer; F:calcium ion binding	2.5	-3.6	-
Ubiquitin-60s ribosomal protein l40	C:extracellular space; F:structural constituent of ribosome; P:protein polyubiquitination; P:stimulatory C-type lectin receptor signaling pathway	-	-4.2	-4.3
60s ribosomal protein l23a-like	C:ribosome; F:structural constituent of ribosome; P:translation	-	-4.0	-
Inosine-uridine preferring nucleoside hydrolase-like	F:hydrolase activity	-3.9	-	-3.3
Cytosolic phospholipase a2	C:intracellular membrane-bounded organelle; F:phospholipase activity; P:phospholipid catabolic process	-	-3.9	-
Actin isoform zwei	C:focal adhesion; F:structural constituent of	-	-3.7	-

	cytoskeleton; P:small GTPase mediated signal transduction; P:cell junction assembly			
Protein phosphatase 1 regulatory subunit 12a-like	C:intracellular part	-	-3.4	-
Ubiquitin isoform cra_e	C:extracellular space; F:protease binding; P:protein polyubiquitination	-	-3.2	-
40s ribosomal protein s3	C:focal adhesion; F:structural constituent of ribosome; P:translational initiation; P:negative regulation of apoptotic process	-	-3.2	-
Tubulin beta chain isoform x1	C:microtubule;F:structural constituent of cytoskeleton;P:microtubule-based process; P:natural killer cell mediated cytotoxicity	-	-	-3.2

1012 \*Fold change (FC) is shown as an average of expression values across replicates. P: Biological Process, C: Cellular  
1013 Component, F: Molecular Function.

**Table 2.** Top differentially expressed genes (FC > |3|) annotated to known proteins in the Manila clam hemocytes challenged with *Perkinsus olseni* trophozoites.

Annotation	GO terms	Log <sub>2</sub> FC*		
		1 h	8 h	24 h
Cytochrome p450 2j2-like	C:intracellular part; F:oxidoreductase activity; P:organic acid metabolic process	-	4.3	-
c-type lysozyme 2	F:lysozyme activity; P:metabolic process	3.9	-	-
Serum amyloid a	C:extracellular space; F:Toll-like receptor 4 binding; P:I-kappaB phosphorylation	3.8	-	-
Cytochrome p450 2h2-like	F:binding; P:metabolic process	-	3.8	-
Cytochrome p450 18a1	C:intracellular membrane-bounded organelle; F:oxidoreductase activity; P:xenobiotic metabolic process	-	3.7	3.1
Early growth response protein 1-b-like	F:nucleic acid binding; F:metal ion binding	-	-	3.7
Apolipoprotein d-like	F:lipid binding; C:extracellular region; P:transport; P:negative regulation of cellular process	-	-	3.6
Ubox domain containing protein	C:ubiquitin ligase complex; P:protein ubiquitination; F:ubiquitin-protein transferase activity	-	-	3.5
NADH dehydrogenase subunit 4	C:mitochondrion; F:NADH dehydrogenase (ubiquinone) activity; P:ATP synthesis coupled electron transport; P:oxidation-reduction process	3.4	-	-
Protein plant cadmium resistance 3-like	-	3.3	-	-
Ubiquitin partial	C:extracellular space; F:protease binding;P:protein polyubiquitination; P:stimulatory C-type lectin receptor signaling pathway	3.2	-	-
Steroid 17-alpha-hydroxylase lyase	F:oxidoreductase activity; F:metal ion binding	-	3.2	-
Cytochrome p450 2j6-like	F:monooxygenase activity;P:oxidation-reduction process	-	3.2	-
Heavy metal-binding protein hip-like	-	3.1	-	-
Complement c1q-like protein 4	F:carbohydrate binding; C:collagen trimer	-	3.1	-
60s ribosomal protein l23a-like	C:ribosome; F:structural constituent of ribosome; P:translation	-	-	-5.5

Alpha skeletal muscle	C:nuclear chromatin; F:protein kinase binding; P:skeletal muscle thin filament assembly	-	-	-5.3
Ubiquitin isoform cra_e	C:extracellular space; F:protease binding; P:protein polyubiquitination	-	-	-5.1
Type i inositol -trisphosphate 5- phosphatase 12 related	F:inositol-polyphosphate 5-phosphatase activity; P:protein dephosphorylation	-	-	-5.1
Actin isoform zwei	C:focal adhesion; F:structural constituent of cytoskeleton; P:small GTPase mediated signal transduction; P:cell junction assembly	-	-	-5.0
Guanine nucleotide-binding protein subunit beta-2-like 1	C:phagocytic cup; F:protein kinase C binding; P:positive regulation of protein phosphorylation; P:activation of cysteine-type endopeptidase activity involved in apoptotic process	-	-	-4.9
40s ribosomal protein s3	C:focal adhesion; F:structural constituent of ribosome; P:translational initiation; P:negative regulation of apoptotic process	-	-	-4.7
Cholesterol 7-alpha-monooxygenase- like	C:endoplasmic reticulum; F:monooxygenase activity; P:steroid metabolic process	-	-	-4.3
Caspase-3-like isoform x2	F:cysteine-type peptidase activity; P:apoptotic process; P:proteolysis	-	-	-3.8
Alpha amylase	F:hydrolase activity, acting on glycosyl bonds; P:carbohydrate metabolic process	-	-	-3.5
Signal transducer and activator of transcription 5b-like	C:intracellular; P:hemopoiesis; P:cell differentiation	-	-	-3.4
Ras-related c3 botulinum toxin substrate 2	C:intracellular part; F:nucleotide binding; P:cellular metabolic process; P:response to stimulus	-	-	-3.3
Interferon regulatory factor 2	F:DNA binding	-	-	-3.1

1016 \* Fold change (FC) was shown as an average of expression values across replicates. P: Biological Process, C: Cellular  
1017 Component, F: Molecular Function.

1018

1019 **Table 3.** Top differentially expressed genes (FC > |3|) annotated to known proteins in the Manila  
1020 clam hemocytes challenged with *Perkinsus olseni* zoospores.  
1021

Annotation	GO terms	Log <sub>2</sub> FC*		
		1h	8h	24h
Tandem repeat galectin	C:immunological synapse; F:protein binding; P:leukocyte chemotaxis	6.3	6.5	-
40s ribosomal protein s16-like protein	C:focal adhesion; F:structural constituent of ribosome; P:rRNA processing;	3.7	-	-
Cytochrome p450 2h2-like	F:binding; P:metabolic process	3.7		4.7
Elongation factor 1- partial	C:cytoplasm; F:translation elongation factor activity; P:translational elongation	-	-	4.3
Eukaryotic initiation factor 4a	F:translation initiation factor activity; P:translational initiation	-	-	3.9
Cytochrome p450 18a1	C:intracellular membrane-bounded organelle; F:oxidoreductase activity; P:xenobiotic metabolic process	3.5	-	-
Cytochrome p450 2j2-like	C:intracellular part; F:oxidoreductase activity; P:organic acid metabolic process	3.3	-	-
Alpha skeletal muscle	C:nuclear chromatin; F:protein kinase binding; P:skeletal muscle thin filament assembly	-10.7	-4.4	-
Ubiquitin isoform cra_e	C:extracellular space; F:protease binding; P:protein polyubiquitination	-10.0	-5.2	-
NADH dehydrogenase subunit 4	C:mitochondrion; F:NADH dehydrogenase (ubiquinone) activity; P:ATP synthesis coupled electron transport; P:oxidation-reduction process	-6.9	-8.2	-
60s ribosomal protein l26	C:large ribosomal subunit; F:structural constituent of ribosome; P:translation	-6.5	-7.5	-
ORF16-lacZ fusion protein		-	-7.3	-
40s ribosomal protein s3	C:focal adhesion; F:structural constituent of ribosome; P:translational initiation; P:negative regulation of apoptotic process	-6.4	-4.6	-
Ubiquitin-60s ribosomal protein l40	C:extracellular space; F:structural constituent of ribosome; P:protein polyubiquitination; P:stimulatory C-type lectin receptor signalling pathway	-	-5.8	-

40s ribosomal protein s16	C:focal adhesion; F:structural constituent of ribosome; P:rRNA processing	-	-5.6	-
Guanine nucleotide-binding protein subunit beta-2-like 1	C:phagocytic cup; F:protein kinase C binding; P:positive regulation of protein phosphorylation; P:activation of cysteine-type endopeptidase activity involved in apoptotic process	-	-5.5	-
Actin isoform zwei	C:focal adhesion; F:structural constituent of cytoskeleton; P:small GTPase mediated signal transduction; P:cell junction assembly	-	-4.7	-
Heavy metal-binding protein hip-like	-	-4.6	-3.8	-
60s ribosomal protein l23a-like	C:ribosome; F:nucleotide binding; P:translation	-	-4.6	-
Complement c1q-like protein 4	F:carbohydrate binding; C:collagen trimer	-4.1	-	-
CALRL protein	C:endoplasmic reticulum; F:calcium ion binding; P:protein folding	-3.8	-	-
Galaxin	-	-3.7	-	-
Serum amyloid a	C:extracellular space; F:Toll-like receptor 4 binding; P:I-kappaB phosphorylation	-3.7	-	-
Ras-related c3 botulinum toxin substrate 2	C:intracellular part; F:nucleotide binding; P:cellular metabolic process; P:response to stimulus	-3.6	-	-
c-type lectin domain family member a	F:monosaccharide binding	-3.5	-	-
Serum amyloid a-5	C:extracellular space; F:chemoattractant activity; P:cell chemotaxis	-3.4	-	-

1022 \* Fold change (FC) was shown as an average of expression values across replicates. P: Biological Process, C: Cellular  
1023 Component, F: Molecular Function.